Neuroscience Letters 609 (2015) 11–17

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research paper

The nuclear retention of transcription factor FOXO3a correlates with a DNA damage response and increased glutamine synthetase expression by astrocytes suggesting a neuroprotective role in the ageing brain

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HIGHLIGHTS

- Nuclear FOXO3a significantly correlates with glutamine synthetase expression.
- FOXO3a nuclear localisation correlates with a DNA damage response.
- Glutamine synthetase expression correlates with increasing Alzheimer pathology.

ARTICLE INFO

Article history:
Received 3 August 2015
Received in revised form 1 October 2015
Accepted 1 October 2015
Available online 8 October 2015

Keywords:
FOXO3a
Glutamine synthetase
DNA damage response
Astrocyte
neurone
Alzheimer's
Pathology
Ageing brain

ABSTRACT

The accumulation of reactive oxygen species leading to oxidative damage and cell death plays an important role in a number of neurodegenerative disorders. FOXO3a, the main isoform of FOXO transcription factors, mediates the cellular response to oxidative stress by regulating the expression of genes involved in DNA repair and glutamine metabolism, including glutamine synthetase (GS). Immunohistochemical investigation of the population-based neuropathology cohort of the Medical Research Council's Cognitive Function and Ageing Study (MRC CFAS) demonstrates that nuclear retention of FOXO3a significantly correlates with a DNA damage response and with GS expression by astrocytes. Furthermore, we show that GS expression correlates with increasing Alzheimer-type pathology in this ageing cohort. Our findings suggest that in response to oxidative stress, the nuclear retention of FOXO3a in astrocytes upregulates expression of GS as a neuroprotective mechanism. However, the activity of GS may be compromised by increasing levels of oxidative stress in the ageing brain resulting in dysfunctional enzyme activity, neuronal excitotoxic damage and cognitive impairment.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia and is pathologically characterised by the extracellular deposition of β-amyloid (Aβ) protein, intracellular neurofibrillary tangles (NFT) of hyperphosphorylated tau, neuronal loss and extensive synaptic changes in the cerebral cortex. Given the currently limited success of Aβ-based therapies [25], it is likely that the successful

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http://dx.doi.org/10.1016/j.neulet.2015.10.001

treatment of AD will also include modulation of mechanisms which protect against other processes causing neuronal dysfunction and neurodegeneration, including excitotoxicity and oxidative stress. Characterisation of these mechanisms is essential to develop novel neuroprotective targets aimed at preventing neuronal dysfunction and cognitive impairment in the ageing population.

Glial pathology occurs in the ageing brain and is a major contributor to age-related neurodegeneration [26,38]. Astrocytes play a key role maintaining homeostasis in the CNS, including the uptake and recycling of neurotransmitters such as glutamate [20]. Extracellular glutamate levels are mainly regulated through re-uptake from the synaptic cleft via glial excitatory amino acid transporters (EAATs) [43]. A functional glutamate-glutamine metabolic cycle between astrocytes and neurones is vital for preventing excessive extracellular accumulation of the neurotransmitter leading to neuronal excitotoxicity [8,11]. Loss of astrocyte-associated EAAT2 and glutamate excitotoxicity are features of brain ageing and neurodegenerative diseases, including AD [18,21,36].

Forkhead box class O (FOXO) proteins form a family of transcription factors which are phosphorylated and regulated by Akt, resulting in their nuclear exclusion and the termination of their activity [42]. Activation of FOXO, depending on cell type, regulates a wide range of biological processes including stress resistance, cell cycle regulation, development and ageing [19]. Glutamine synthetase (GS), which catalyses the conversion of glutamate to glutamine, is highly expressed in astrocytes and is transcriptionally regulated by the phosphoinositide-3-kinase (PI3K)-Akt-FOXO pathway [41]. In contrast to Akt signalling, oxidative stress induces the nuclear retention of FOXO which, depending on the severity of the stimulus, results in either apoptosis or a protective response, including the transcription of anti-oxidant genes and activation of a DNA damage response [3,15,17,39]. One member of the forkhead transcription factors, FOXO3a, has been implicated in a number of neurodegenerative disorders, including AD [3,29,33,45], motor neuron disease [24], Parkinson's disease [12] and stroke [13], and is expressed throughout the cortex and hippocampus [14].

The Medical Research Council's Cognitive Function and Ageing Study (CFAS) is a well characterised prospective, longitudinal, population-based neuropathological study of the aging population (over 65 yrs) [44]. Studies performed on this population-representative cohort we have previously demonstrated a reduction in EAAT2 expression [36] and down-regulation of the PI3K-Akt pathway by astrocytes associated with increasing levels of Alzheimer-type pathology [37], and quantitated oxidative stress and the associated DNA damage response in this ageing cohort [35]. Given the proposed role of excitotoxicity in age-related neurodegeneration, we have now investigated the subcellular localisation of FOXO3a and its correlation with GS expression, astrogliosis and the DNA damage response in the ageing brain.

2. Materials and methods

2.1. Human CNS cases

Human autopsy brain tissue was obtained from one centre of the Medical Research Council Cognitive Function and Ageing Study (MRC CFAS) [1,44], following multi-centre research ethics committee (REC) approval (REC Reference number 11/H0308/2). Neuropathological lesions were assessed as part of the core CFAS neuropathology study using a modified protocol from the Consortium to Establish a Registry of Alzheimer's Disease (CERAD) [23] (wwws.cfas.ac.uk) and Braak neurofibrillary tangle staging [7]. The cases were categorised into groups representing entorhinal stages (Braak stages 0-II; 30 cases), limbic stages (Braak stages III-IV; 50 cases) and isocortical stages of tangle pathology (Braak stages V-VI; 17 cases). The mean age of death was 85.6 (SEM 7.4) years. Dementia status at death had been previously determined, based on all information available for each participant, including algorithmic (AGECAT) assessment in life, information from death certification and a Retrospective Informant Interview (RINI) developed by CFAS [34]. 59 participants had clinical dementia, 37 did not and in 2 cases clinical dementia status was undetermined. The median post-mortem delay was 17 h (IQR 10–32 h) and brain pH 6.49 (IQR 6.25–6.75). Formalin-fixed and frozen lateral temporal cortex samples (superior/middle temporal gyrus, Brodmann areas 22/21) were available for all cases and were used in the immunohistochemistry and western blotting experiments, respectively. The neuronal and astrocyte DNA damage response (DDR) (γH2AX and DNA-PKcs nuclear immunoreactivity), astrogliosis (GFAP immunoreactivity), and local measures of AD-type pathology (Aβ and AT8) were previously assessed in these cases [35,36]. A total of 98 participants were included in these analyses, where 61 are females.

2.2. Immunohistochemistry

Immunohistochemistry was performed using a standard avidin-biotin complex (ABC) method. Sections were deparaffinised, rehydrated to water and endogenous peroxidase activity quenched by placing the sections in 0.3% H_2O_2/methanol for 20 min at room temperature (RT). Sections were subjected to antigen retrieval (0.01 M tri-sodium citrate pH 6.5, pressure cooker). Following incubation with 1.5% normal serum for 30 min at RT, the sections were incubated overnight at 4 °C with the well characterised, commercially available antibodies against FOXO3a (1:100; AbCam, UK), or glutamine synthetase (1:500; Millipore, UK). As phosphorylation of FOXO3a leads to the nuclear exclusion of the transcription factor and the termination of its activity, we elected to use an antibody which was raised against the N-terminus of the protein, as opposed to an antibody to specifically detect the phosphorylated form. To visualise antibody binding, the horse-radish peroxidase avidin biotin complex was used (Vectastain Elite kit, Vector Laboratories, UK) with 3,3′-diaminobenzidine (DAB) as the chromagen (Vector Laboratories, UK; brown).

To investigate astrocyte association with FOXO3a, dual labelling with the astrocyte marker GFAP was performed. Following incubation with the avidin-biotin blocking kit (Vector Laboratories, UK), FOXO3A immunostained sections were incubated overnight at 4 °C with anti-GFAP (1:500; DakoCytomation, UK), followed by the alkaline-phosphatase-conjugated avidin-biotin complex (Vectastain Elite kit, Vector Laboratories, UK), developed with alkaline phosphatase substrate 1 (Vector Laboratories, UK; red) and lightly counterstained with Mayer's haematoxylin. Negative controls, either omission of the primary antibody or isotype controls, were included in every run.

2.3. Quantitative analysis of FOXO3a and GS

Assessment of FOXO3a and GS-specific immunoreactivity was performed by capturing bright-field microscopic images in 3 adjacent 350 μm-wide cortical ribbons, consisting of contiguous fields to cover the total cortical thickness through the apex of the gyrus, using a x20 objective (Nikon Eclipse Ni-U microscope, Nikon, UK) and analysed using the Analysis D̂ software (Olympus Biosystems, Watford, UK). For GS, the image was thresholded and the immunoreactive area of the field determined per total area examined. The number of FOXO3a positive pyramidal neuronal nuclei was determined using a size exclusion of >450 pixels, and the number of positive glial nuclei determined by subtracting the number of pyramidal neuronal nuclei from the total number of positive nuclei.